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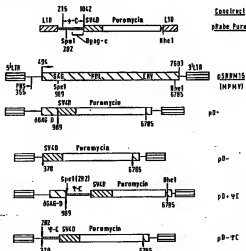
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ :		(11) International Publication Number:	WO 94/03622
C12N 15/86, 7/04, 5/10	**A1**	(43) International Publication Date:	17 February 1994 (17.02.94)

(21) International Application Number: PCT/GB93/01620

(22) International Filing Date: 30 July 1993 (30.07.93)

(30) Priority data:
9216324.5   31 July 1992 (31.07.92)   GB

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(81) Designated States: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: D-TYPE RETROVIRAL VECTORS, BASED ON MPMV

(57) Abstract

Retroviral vectors are based on a packaging sequence of a D-type retrovirus and are useful as heterologous gene delivery systems in gene therapy. D-type retrovirus do not share the oncogenic characteristics of the more conventionally used C-type retroviruses, and have a high packaging efficiency.

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D - TYPE RETROVIRAL VECTORS, BASED ON MPMV.

This invention relates to retroviral vectors. In
particular, the invention relates to retroviral vectors
which have reduced oncogenic risk, which is particularly
relevant when used in the field of gene therapy, and
packaging efficiencies comparable to natural viruses.

There has been much recent activity towards the idea of
correcting genetic disease in humans or, in principle at
least, in other animals by "gene therapy". As various
review articles have pointed out (Weatherall *Nature* 349
275-6 (1991); Miller *Blood* 76(2) 271-8 (1990); Friedmann
Science 244 1275-81 (1989) and Anderson *Science* 226 401-9
(1984)), progress has been slower than the amount of
activity might suggest. One of the problems has been to
devise a competent, safe and reliable method for
introducing heterologous genetic material into the genome
of target cells.

The use of retroviruses as vehicles for gene transfer is
a promising approach. Unlike all other viruses,
retroviruses replicate their RNA genome via a DNA
intermediate that becomes permanently integrated into
host cell DNA. Retroviruses are widespread in nature,
and the many hundreds of isolates can be classified into
groups according to biological properties, morphology and
genome structure. Four types of particles, A, B, C and
D, have been identified by electron microscopy. Most of
the cancer-causing viruses that have been studied
extensively are C-type oncoviruses, although the
important mouse mammary tumour viruses have the B-type
morphology. The C-type virus group itself consists of a
vast array of viruses.

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C-type oncoviruses have been the centre of much extensive
study both biologically and molecularly.   This has
resulted in the development of several generations of C-
type retroviral vector sand packaging cell lines, derived
5   from both murine and avian viruses (Cosset et al,
J. Virol. 64 1070-1078 (1990) and Danos and Mulligan
Proc. Natl. Acad. Sci. USA, 85, 6460-6460 (1988)). These
vectors generate recombinant retroviruses capable of
delivering foreign genes into cells more efficiently than
10   other physical means of gene transfer.   Generation of
these vectors has required the definition of the cis- and
trans- acting functions required for the production of
recombinant retrovirus particles (Miller et al, Somatic
Cell. Mol. Genet. 12, 175-183 (1986)). In particular,
15   the most basic vectors must include the two viral long
terminal repeats (LTRs), sequences required for reverse
transcription (which lie both within and adjacent to the
5' and 3' LTRs), and an RNA packaging sequence which is
required for encapsidation of viral genomes into viral
20   particles (Swanstrom and Vogt, Current Topics in
Microbiology and Immunology, 157 (1990)).   The viral
packaging sequence is required for the dimerisation of
the two genomic length RNA molecules (Bieth et al,
Nucleic Acids Res. 18 119-127 (1990) and Prats et al,
25   J. Virol. 64, 774-783 (1990)) which are subsequently
directed into the maturing diploid viral particle
following binding by a gag-encoded zinc-finger protein
(Leis and Jentoft J. Virol. 48, 361-369 (1983)). In the
case of the C-type murine leukaemia viruses (MLV), the
30   most effective packaging sequence lies just downstream of
the region containing both the 5' splice donor site and
the binding site for the tRNA primer (PBS) (which is used
in reverse transcription for the initiation of minus
strand DNA synthesis), and also includes a portion of the

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gag gene (Bender *et al*, *J. Virol.* **61**, 1639-1646 (1987)).
Packaging sequences have also been defined for the Human
Immunodeficiency virus Type 1 (Lever *et al*, *J.Virol.* **62**,
4085-4087 (1989)) (Watanabe and Temin *Proc. Natl. Acad.*

5   *Sci. USA*, **79**, 5986-5990 (1982)) and Avian Leukosis Virus
(ALV) (Watanabe and Temin *Proc. Natl. Acad. Sci. USA*, **79**,
5986-5990 (1982)).

C-type vectors have proved of great value in defining
10   aspects of the retroviral life cycle (Panganiban and
Fiore *Science* **241**, 1064-1069 (1988)), in the
identification of retroviral receptors (Albritton *et al*,
Cell **57**, 659-666 (1989), O'Hara *et al*, *Cell Growth and*
Differentiation, **1** 119-127 (1990) and Takeuchi *et al*,
15   *J. Virol.* 1219-1222 (1992)), and even in approaches to
gene therapy of human disease (Rosenberg *et al*, *N. Engl.*
J. Med. **323** 570-578 (1990)). However, various
limitations of C-type retroviral vectors have become
apparent, especially in the field of gene therapy, partly
20   because of the continual risk associated with these
potentially oncogenic vectors (Temin H.M., *Science* **246**,
983 (1989)). Further, the host range for target cells is
somewhat limiting: in particular, vectors more effective
at infecting stem cell populations would be useful.
25   Therefore, a different type of viral vector (retroviral
or otherwise) is sought as an alternative to C-type
vectors in gene transfer protocols.

It has now been realised that D-type retroviruses may
30   form the basis of highly efficient vectors which do not
suffer from all the disadvantages of those based on C-
type retroviruses. This realisation is based on the
discovery of a packaging sequence for D-type
retroviruses.

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Mason-Pfizer monkey virus (MPMV) is the prototype D-type
retrovirus of the oncovirus family of retroviruses. The
first isolate was derived from a female rhesus monkey
(*M. mulatta*) with a breast carcinoma (Chopra and Mason,
Cancer Res. 30, 2081-2086 (1970)) and MPMV has now been
molecularly cloned and sequenced (Sonigo *et al.*, *Cell* 45
375-385 (1986)). Subsequent studies have failed to
support any further link between this class of
retroviruses and cancer. MPMV infection is, however,
associated both with a T cell immunosuppression in young
rhesus monkeys (Fine *et al*, *Natl. Cancer Inst.* 54, 651-
658 (1975)) and with the simian acquired immuno-
deficiency syndrome (SAIDS) in adult rhesus monkeys
(Stromberg *et al*, *Science* 224 289-292 (1984)). A second
D-type retrovirus serotype was isolated from a Formosan
rock macaque (*M. cyclopis*) also suffering from SAIDS
(Marx *et al*, *Science* 223, 1083-1086 (1984)), and
subsequently other SAIDS retrovirus serotypes (SRV) have
been isolated (Marx *et al*, *Science* 223 1083-1086 (1984),
Daniel *et al*, *Science* 223 602-605 (1984), Marx *et al*,
J. Virol 56 571-578 (1985) and Sommerfelt *et al*,
J. Virol. 64 6214-6220 (1990)), including SRV-5. D-type
retroviral infection in humans has not yet been linked
with any disease.

According to a first aspect of the present invention,
there is provided a recombinant or isolated RNA
comprising a D-type retroviral packaging sequence or a
recombinant or isolated DNA corresponding to or
complimentary to such an RNA.

The D-type retroviral packaging sequence may be identical
to a natural sequence, which may be isolated from a D-
type retrovirus. An example of a D-type retrovirus is

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Mason-Pfizer monkey virus (MPMV), the packaging sequence of which extends downstream of the tRNA^{lys} primer binding site (PBS^{lys}), at position 365 of the MPMV genome (using the numbering system of pSHRM15, an infectious molecular

5   clone of MPMV (Sonigo et al., Cell 45 375-385 (1986) and Rhee et al., J. Virol. 64 3844-3852 (1990))). It has been established, in the accomplishment of the present invention, that the 619 bases downstream of the PBS^{lys} are sufficient to confer packaging activity; however, it is

10   possible that not all that sequence is necessary, and so not all of it need be present.

Fine detail mapping of the sequence, and site-directed mutagenesis, will further delineate the precise 5' and 3'

15   boundaries of the natural D-type packaging sequence. It would be predicted that the 5' extent of this sequence will lie downstream of the 5' splice donor site of MPMV, so that only full length genomic transcripts, rather than spliced sub-genomic RNA molecules, will be efficiently

20   packaged. A putative splice donor signal (5'-AGGT-3') lies, upstream of the PBS^{lys}, at positions 312-315. Since any vector must retain the PBS for reverse transcription to occur (Swanstrom and Vogt, Current Topics in Microbiology and Immunology, 157 (1990), this sequence

25   element effectively, therefore, represents the 5' boundary of the packaging sequence. With respect to the 3' boundary, however, further deletion analysis will be required to define the minimal natural 'core' sequence which is both necessary and sufficient for viral

30   packaging.

As with all the features of this invention, the above sequence may be, and preferably is, identical to a natural sequence. However, it will be appreciated that

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sequences which are not identical to natural sequences
may work perfectly adequately and may even in some cases
be better; for this reason their use is not ruled out.
The invention therefore encompasses sequences which have
5   qualitatively the same (relevant) function as a natural
sequence but which have different base compositions.
There will usually be homology between natural sequences
and other sequences having qualitatively the same
function; this homology may be at least 80% or even at
10   least 90%, 95% or 99%, in increasing order of preference.

Alternatively or in addition, sequences useful in the
invention may hybridise to a natural sequence whose
function is sought. Hybridisation may be under stringent
15   conditions (see Maniatis et al., "Molecular Cloning: A
laboratory Manual", Cold Spring Harbor Laboratory (1982),
pp 387-389). An example of stringent hybridisation
conditions is hybridisation at 4xSSC at 65°C, followed by
washing in 0.1xSSC at 65°C for one hour. An alternative
20   exemplary hybridisation condition is 50% formamide, 4xSSC
at 42°C.

If hybridisation is not under stringent conditions, it
may be under relaxed conditions. Examples of such non-
25   stringent hybridisation conditions are 4xSSC at 50°C or
hybridisation with 30-40% formamide at 42°C.

The D-type retroviral packaging sequence is useful in the
construction of viral vectors. According to a second
30   aspect of the invention, there is provided a retroviral
vector, or a DNA construct having a strand corresponding
or complementary to a retroviral vector, comprising: (a)
sequence capable of providing retroviral long terminal
repeats (LTRs); (b) sequence required for reverse

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transcription; and (c) a D-type retroviral packaging sequence.

5   Long terminal repeats (LTRs) are present on double stranded DNA reverse-transcribed from retroviral genome RNA. A DNA construct in accordance with the invention will include the LTRs necessary for host cell genome incorporation and expression. The LTRs are not present
10   as such in retroviral genomic RNA; instead, the genomic RNA contains sequences from a combination of which the LTRs are derived. Specifically, each LTR (which is usually in the order of 300 to 1000 long) is generally derived from a combination of sequences present at the 3'
15   end (U_3), the 5' end (U_5) or both ends (R) of the RNA genome and has the structure U_3-R-U_5. Following synthesis, the viral DNA is integrated into cellular DNA so that the ends of the LTRs are directly joined to cellular sequences to form a stable structure, the provirus.

20   In the present invention, the LTR sequences will generally be derived from one or more D-type retroviruses such as MPMV.

        Secondly, vectors in accordance with the invention also
25   comprise sequence necessary for reverse transcription. Such sequence does not necessarily include sequence coding for the enzyme reverse transcriptase. Rather, retrovirus provirus DNA contains a replication initiation site, in fact generally one on each strand of the double
30   stranded DNA molecule. On the minus strand, the replication initiation site is designated r^- and is the tRNA binding site (RBS); on the plus strand, the replication initiation site is designated r^+ and is otherwise known as the purine-rich site.

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In the present invention, the sequence necessary for reverse transcription will generally be derived from one or more D-type retroviruses such as MPMV.

5   Thirdly, vectors in accordance with the invention comprise a D-type retroviral packaging sequence, as described above.

Donor (D) and acceptor (A) splice sites may also be
10   present. D and A sites are used naturally *in vivo* for expression of retroviral genes from spliced subgenomic RNA (mainly *env*). In retroviral vectors, such as provided by the present invention, they enable the construction of "double expression vectors" which can
15   express two, rather than one, heterologous inserted nucleic acid sequence. As such, D and A sites are not themselves directly involved in retroviral packaging of the genomic RNAs.

20   These components of the vectors of the invention constitute the minimum needed for transcription and transmission of the viral genome. Such minimal vectors would not include sequences corresponding to the viral genes *gag* (coding for the group-specific (that is, viral
25   core) antigens), *pol* (RNA-dependent DNA polymerase (reverse transcriptase)) or *env* (envelope proteins). In fact, the absence of one or more or even all three of such genes may be preferred, so that the indefinite production of infectious virus particles, once the
30   proviral DNA in integrated with the host cellular DNA, is avoided.

D-type vectors defective or, ideally, completely deleted in *env* are particularly advantageous as there will be no

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functional envelope gene transferred to target cells.
This may reduce the risk of immunosuppression, the major
pathogenic activity of the D-type retroviruses.

5   Vectors or constructs in accordance with the invention
will often contain exogenous DNA (or RNA corresponding to
it, as the case may be). For use in gene therapy, a
vector in accordance with the invention will contain the
exogenous gene or other DNA which it is desired to
10   transfer to the intended recipient. Exogenous DNA may
code for the replacement or substitute of a defective or
missing enzyme or other protein in the patient (whether
human or animal). The enzyme or other protein may
normally function within a cell or circulate round the
15   body; examples of circulating proteins include hormones
and blood factors. Genes coding for proteins whose levels
do not have to be controlled precisely and/or genes which
can cause disease with a single defect are attractive
candidates for the invention. Examples include:

20
        Haemophilias
            Factor VIII deficiency
            Factor IX deficiency
        Immunodeficiencies
25   Adenoside deaminase deficiency
            Purine nucleoside phosphorylase deficiency
        Urea cycle disorders
            Ornithine transcarbamylase deficiency
            Arginosuccinate synthetase deficiency
30   Anaemias
            β-thalassaemia
            Sickle cell anaemia
        Emphysema
            α_1-antitrypsin deficiency

Lysosomal storage disorders
        Glucocerebrosidase deficiency
Other metabolic disorders
        Phenylalanine hydroxylase deficiency
5   Hypoxanthine-guanine phosphoribosyl transferase
                deficiency
Cancers
        Cytokine genes
        Cytotoxic genes
10   MMC genes
        Tumour suppressor genes
        Antisense genes

Exogenous DNA may include complementary, genomic or
15 synthetic DNA coding for a protein of interest.   In
    addition, regulatory sequences may be present.   For
    example, DNA coding for a protein of interest may be
    under the control of a suitable promoter.   In some
    embodiments of the invention, only control sequences may
20 be present if the intention is, for example, to "rescue"
    the expression of genes present but not correctly
    functioning in the intended recipient.

    The exogenous DNA may comprise a suitable marker DNA
25 sequence; this may be primarily for investigative or
    experimental purposes, but a suitable selectable marker
    may enable infective particles containing retroviral
    vector RNA to be selected, as will be described below.
    Many selectable markers are based on antibiotic
30 resistance; an example is puromycin resistance.   Others
    include geneticin, L-histidinol, hygromycin B, gpt,
    thymidine kinase and/or APRT.

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It is to be stressed that the nature of the exogenous DNA
will depend entirely on the intended purpose of the
retroviral vectors of the invention, and so is not
particularly limited.

5

To be useful in delivering its genetic payload, whatever
it may be, to a target cell, a retroviral vector must be
rendered infective. This is usually achieved by means of
a helper virus, using an appropriate packaging host cell.
10   For example, a DNA construct in accordance with the
invention is introduced into a packaging host cell which
is a suitable host for the helper virus (such as HeLa for
MPMV or SRV-5) by any suitable transfection method, such
as calcium phosphate precipitation. Existing methods and
15   materials known in the art may be used for these
purposes; a useful summary can be found in "Methods in
Molecular Biology, Vol. 8: Practical Molecular Virology:
Viral Vectors for Gene Expression", particularly Chapter
1 ("The Retroviral Life Cycle and the Molecular
20   Construction of Retrovirus Vectors", by Richard Vile) and
Chapter 5 ("Selectable Markers for Eukaryotic Cells",
also by Richard Vile). This book, particularly the
chapters referred to above, and the references cited
therein, are herein incorporated by reference.

25

Host cells transfected with DNA constructs as described
above and infectious particles containing retroviral
vectors as described above are also within the scope of
the invention.

30

The viral particles would be used to infect target cells
(ie. those usually which are defective in expression of
the deviant gene or those which can act to secrete the
relevant protein) either ex vivo followed by replacement

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of the infected cells into the patient or, conceivably,
in vivo directly.

5   Stem cells are the ideal target so that the "defective"
     cell population can be rendered non defective by
     permanent division and replenishment of converted cells
     from the altered stem cell pool. The problem for all
     cell lineages is to identify, infect and purify the
     relatively sparse stem cells from the total cell
10   population. Stem cells do not have to be infected – ie,
     dividing cells of the mature lineage could be infected by
     the retroviruses. However, if stem cells are not
     infected continual, repeated therapy will be required
     (possibly) because the corrected cell population will be
15   lost without replenishment from any "corrected" self-
     replicating stem cells.

     The particles can be used as markers for infection both
     in vivo (ie. cell lineage determination etc) or *in vitro*
20   (ie. receptor identification studies).

     The invention will now be illustrated by the following
     Example. The Example refers to the accompanying drawing,
     in which:

25

          FIGURE 1 shows the molecular synthesis of constructs
          A, B, C and D from pSHRM15 and pBabe Puro. The
          following points of notation apply:
          Ψ-C refers to the extended packaging sequence of
30   MoMLV (Bender *et al.*, 1987);
          Numbering in A to D refers to the junction
          nucleotide of pSHRM15 preceding/succeeding
          sequences of pBabe Puro (Sonigo *et al.*, 1986);

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> *Numbering in italics* refers to nucleotide sequence
> of MoMLV (Weiss et al., 1985);
>
> ∂GAG-D refers to the first 495bp of MPMV GAG gene;
>
> ∂GAG-c refers to the first 342bp of Mo-MLV GAG gene;
> and

5

> PBS   refers to the tRNA binding site of MPMV (Sonigo
> et al., 1986)

<u>EXAMPLE</u>

10

The packaging sequence of MPMV was localised by
constructing four separate recombinant proviral genomes
(Figure 1). A, [p(∂gag^D)$^+$], was generated by ligating an
15 SV40-Puromycin selectable marker cassette between the
*Spe*I (position 989) and *Nhe*I (position 675) restriction
sites in pSHRM15, an infectious molecular clone of MPMV,
(Sonigo *et al*, *Cell* 45 375-385 (1986) and Rhee et al,
J. Virol. 64, 3844-3852 (1990)). In principle, any
20 infectious molecular clone of MPMV could be used. As the
sequence of MPMV has been published (Sonigo et al, (loc.
cit.), polymerase chain reaction (PCR) technology may be
used to clone it. The selectable marker cassette was
derived from the C-type retroviral vector, pBabe Puro
25 (Morgernstern and Land *Nucleic Acids Res.* 18, 3587-3596
(1990)), by PCR amplification in which artificial *Spe*I
and *Nhe*I sites were incorporated into the 5′ and 3′
primers respectively. Hence, A retains 624 bp downstream
from the tRNA^{lys} primer binding site (PBS^{lys}) (position
30 365). This includes 495 bp of the *gag* gene but none of
pol and only a small part of *env* (818 bp). In construct
B, [p(∂gag)$^-$]; the SV40 puromycin cassette of pBabe Puro
was amplified with primers which allowed it to be cloned
into pSHRM15 at position 370, just downstream, but
including all of, the PBS^{lys}. Therefore, B lacks 619 bp

of sequence retained in construct A. To generate C,
[p(∂gag^D)$^+$(Ψ^C)], the SpeI-NheI fragment of pBabe Puro was
cloned between the SpeI and NheI sites of pSHRM15. This
fragment of pBabe Puro includes most of the extended
packaging sequence (Ψ+) (760 bp out of 827 bp) of the C-
type Moloney Murine leukaemia virus (Mo-MLV) (14, 24), as
well as the SV40 puromycin cassette. Finally, for D,
[p(∂gag)$^-$(Ψ^C)], this same SpeI-NheI fragment of pBabe Puro
was amplified using PCR primers permitting it to be
cloned into the same site (position 370) as for construct
B - that is just downstream from the PBS^{lys}.

All four of these D-type constructs, along with pBabe
Puro, were transfected separately into HeLa cells, as
well as into the Murine Leukaemia Virus amphotropic (MLV-
A) and ecotropic (MLV-E) C-type packaging cell lines
PA317 and Ψ2 (Miller and Buttimore Mol. Cell Biol. 6,
2895-2902 (1986) and Mann et al, Cell 33 153-159 (1983))
using the calcium phosphate precipitation technique (Chen
and Okayama Mol. Cell Biol. 7 2745-2752 (1987)).
Transfected clones were selected in puromycin (sigma) at
1.25μg/ml and approximately 100 clones from each
transfection were pooled and maintained in selection.

10^5 cells of each of the five populations of transfected
HeLa cells, along with an untransfected control, were
then mock infected or infected with either wild type MPMV
or the D-type retrovirus (SRV-5 (Daniel et al, Science
223 602-605 (1984)), using cell-free supernatants
(0.45μm-filtered) from virus producing cells. After 10-
14 days, syncytia were clearly observed exclusively in
the infected cell populations. Cell-free supernatants
were harvested and used to infect fresh HeLa or NIH 3T3
cells in the presence of polybrene (Aldrich) at 4μg/ml.

15

Two days after infection, the target cells were split
into puromycin selection and three weeks later surviving
colonies were counted. Similarly, cell free supernatants
were harvested directly from 10^7 cells of the
transfected/selected populations of PA317 and Ψ2 cells
and used to infect target HeLa or NIH3T3 cells which were
also then selected in puromycin. Any titre of puromycin
resistant colonies would indicate that a vector genome
had been rescued from HeLa, PA317 or Ψ2 cells lines by
the relevant wild type virus (MPMV, SRV-5, MLV-A or MLV-
E). For this to occur, packaging of the vector genomic
length RNA transcripts into capsid particles, reverse
transcription of the RNA into DNA and productive
integration of the resulting DNA provirus into the target
cell would all be required. The results of these rescue
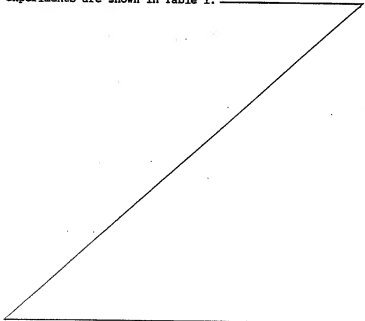experiments are shown in Table 1.

Table 1: Detection of recombinant MPMV or SRV-5 virus following rescue of vectors carrying a D-type packaging sequence.

Construct Transfected	Target Cell Transfected	Titre of Recombinant Virus Released Following Rescue by Wild Type Virus - Puromycin C.F.U./ml. (Titre of Wild Type MPMV co-released from transfected cells). (syncytial inducing units/ml.)							
		Rescuing Virus:							
		MPMV		SRV-5		MLV-A		MLV-E	
	Timing Cell:	HeLa	3T3	HeLa	3T3	HeLa	3T3	HeLa	3T3
None	HeLa	0 (10⁴)	0	0	0	–	–	–	–
A	PA317	–				0	0	–	–
	Ψ2	–				–	–	0	0
	HeLa	1.2x10² (10²)	0	80	0	–	–	–	–
B or D	PA317	–				0	0	–	–
	Ψ2	–				–	–	0	0
	HeLa	0 (10³)	0	0	0	–	–	–	–
C	PA317	–				0	0	–	–
	Ψ2	–				–	–	0	0
	HeLa	1.0x10² (10²)	0	80	0	–	–	–	–
pBabe Puro	PA317	–				0	0	–	–
	Ψ2	–				–	–	0	0
	HeLa	0 (10³)	0	0	0	–	–	–	–
	PA317	–				2.8x10³	1x10⁴	–	–
	Ψ2	–				–	–	0	2x10⁵

Titre values represent the average number of puromycin-resistant colonies produced/ml of cell-free supernatant, harvested from approximately 10⁷ cells infected with ~10³ virus particles of MPMV or SRV-5 10-14 days previously or from ~10⁷ PA317 or Ψ2 cells transfected with the appropriate vector. Supernatant recovered from all the transfected cells lines gave no titre on either HeLa or 3T3 cells in the absence of infection with a wild type virus. Values shown are the mean of at least two separate experiments.

The symbol "-" means "not done".

Whereas both vectors A and C could be efficiently rescued
from transfected HeLa cells by both D-type viruses,
neither construct B nor D were rescued. None of the D-
type constructs could be rescued by the C-type viruses
5   MLV-A or MLV-E, although, as expected, the C-type vector
pBabe Puro was packaged to high titre. Similarly, pBabe
Puro could not be rescued by either d-type virus from
HeLa cells.

10   To confirm that the virus rescued by MPMV infection of
HeLa cells transfected by constructs A or C are genuinely
MPMV recombinants, receptor interference and virus
neutralisation studies were carried out. Supernatant
from HeLa/A transfectants infected with MPMV was used to
15   infect human Raji cells or Raji cells productively
infected with MPMV or SRV-5. Whereas the Raji cells
survived selection in puromycin following infection (at
a titre of recombinant virus of 10^2 puromycin-resistant
cfu/ml), no survivors were seen following infection of
20   Raji/MPMV or Raji/SRV-5 with the recombinant stocks and
selection in puromycin (Table 2).

Table 2: Cell tropism and demonstration of receptor
interference of recombinant MPMV virus stocks.

25

Target Cell Line for Infection	Number of Puromycin-resistant colonies (/ml of virus supernatant).	
	Source of supernatant: HeLa/A rescued by MPMV	HeLa
Hela	1.3×10^2	0
NIH 3T3	0	0
Raji	1.0×10^2	0
Raji/MPMV	0	0
Raji/SRV-5	0	0

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This demonstration of receptor interference (Sommerfelt and Weiss *Virology* 176 58-69 (1990), Steck and Rubin *Virology* 29 628-641 (1966) and Steck and Rubin *Virology* 29 642-653 (1966)) reflects the inability of one virus to infect a cell which is already infected by a virus which uses the same cellular receptor. Therefore, the recombinant virus recovered from HeLa/A transfected cells recognises the same cellular receptor as both MMV and SRV-5 on Raji cells. It has already been demonstrated that these two D-type viruses utilise a common receptor (Sommerfelt and Weiss, *loc. cit.*). Furthermore, the recombinant viral stock was completely and specifically, neutralise by preincubation with anti-MPMV rabbit antiserum, whereas a normal rabbit serum or normal culture medium were both unable to neutralise the recombinant virus when it was plated on HeLa cells (Table 3). The neutralising anti-serum was, however, unable to block infection of HeLa cells by the C-type virus released by PA317 cells transfected with p Babe Puro (Table 3).

Table 3: Neutralisation of recombinant MPMV Puromycin virus.

Supernatant	Treatment of supernatant prior to infection:	Titre of treated supernatant on HeLa cells:	
HeLa/A transfectants rescued with MPMV	Rabbit anti-MNPMV serum*	0	(puro c.f.u./ml)
	Normal rabbit serum*	90	"
	Culture medium*	1.1×10^1	"
PA317/pBabe Puro transfectants	Rabbit anti-MPMV serum*	3×10^3	"
	Normal rabbit serum*	1×10^3	"
	Culture medium*	3×10^3	"

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*Virus stocks were incubated with a 1:20 dilution of sera or culture medium alone for 60 minutes at 37°C and were then plated on HeLa cells.

5   Taken together, these data confirm that the recombinant virus rescued from HeLa/A transfectants have the envelope specificity of MPMV rather than of any endogenous retrovirus which might have been activate in the transfected HeLa cells.

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Therefore, only recombinant D-type genomes which retained the 619 bp fragment of MPMV lying downstream of the PBS^{lys} could be rescued from transfected HeLa cells by D-type retroviruses (constructs A and C). When this sequence
15   was absent (construct B) or replaced by 760 bp of sequence derived from the packaging signal of a C-type retrovirus, the vector was also no longer rescued by D-type viruses (construct D). The packeagable recombinant genomes (A and C) appear to be able to compete for
20   packaging with wild type genomes since any titre of recombinants appeared to reduce the levels of wild type virus released from the producer cells (Table 1). When no recombinant genomes, or genomes that could not be rescued, were present in HeLa cells, the titre of wild
25   type virus that could be generated from these producer cells was generally increased. This would suggest that the vectors A or C might be rescued to higher titres than those observed here if a helper-free D-type packaging cell line were used in which there are no (wild-type)
30   genomes competing for packaging. Interestingly, SRV-5 was able to rescue both MPMV-derived constructs at similar levels to MPMV, suggesting that there is a high degree of cross-recognition of this putative packaging sequence within the D-type retroviruses. This

heterologous recognition between viruses of the same type
probably occurs, therefore, at the level of interaction
of the (gag-encoded) viral packaging protein (Leis and
Jentoft J. Virol. 48, 361-369 (1983)) with the secondary
5   structure formed in the RNA dimer.

Previous work has shown that a range of C-type viruses
can rescue a C-type vector from a human HOS cell line
(Takeuchi et al, Virology 186 792-294 (1992)) but the C-
10 type vector could not be rescued by a D-type virus. The
data in the present in invention confirm these findings
since pBabe Puro could not be rescued by either MPMV or
SERV-5 from the human HeLa cell line. It has now been
shown that a similar, reciprocal relationship exists -
15 that is that two D-type viruses can rescue a D-type
vector but that C-type viruses cannot cross-rescue the
vector (constructs A and C could not be packaged by PA317
or Ψ2 cells). Moreover, the presence of most of the
extended C-type Ξ packaging sequence (Bender et al,
20 J. Virol. 61, 1639-1646 (1987)) was insufficient to allow
rescue of an otherwise D-type vector by MPMV or SRV-5
(construct D). This would suggest that a stringent
selection operates in viral packaging at the level of
recognition of the secondary structure formed by the RNA
25 packaging sequence with heterologous viral capsid
proteins (Leis & Jentoft, loc. cit. and Takeuchi et al.,
loc. cit.). Additionally, rescue of a recombinant genome
by wild type virus requires compatibility of the cis-
acting genome sequences with the viral structural and
30 enzymatic proteins for all stages in the viral life
cycle, not just at the stage of viral packaging. This is
shown here by the inability of the C-type packaging
sequence (either alone, or in tandem with a D-type
sequence) to allow rescue of an otherwise D-type genome

by wild type C-type (Constructs C and D). Presumably,
the C-type reverse transcriptase- and integrase-
associated proteins are unable to recognise the D-type
genomic sequences in the vectors. Therefore, by analogy
5   with the position of the Ψ sequence in other retroviruses
(Bender *loc. cit.*, Lever *et al*, *J.Virol*. **62**, 4085-4087
(1989) and Watanabe and Temin *Proc. Natl. Acad. Sci. USA*,
79, 5986-5990 (1982)) the results shown here suggest that
the 619 bp fragment of MPMV identified here as necessary
10   for efficient rescue represents a generalised D-type
retroviral packaging sequence. (A role for this sequence
in D-type specific reverse transcription and/or
integration cannot, however, be excluded at this stage.

CLAIMS

1.   A recombinant or isolated RNA comprising a D-type retroviral packaging sequence or a recombinant or isolated DNA corresponding to or complimentary to such an RNA.

2.   An RNA or DNA as claimed in claim 1, wherein the D-type retroviral packaging sequence is identical to a natural sequence.

3.   An RNA or DNA as claimed in claim 2, wherein the D-type retroviral packaging sequence is derived from Mason-Pfizer monkey virus (MPMV).

4.   An RNA or DNA as claimed in claim 3, wherein the packaging sequence comprises sequence corresponding to the 619 bases downstream of the tRNA^{lys} primer binding site (PBS^{lys}), at position 365 of the MPMV genome (using the numbering system of pSHRM15).

5.   A retroviral vector, or a DNA construct having a strand corresponding or complementary to a retroviral vector, comprising: (a) sequence capable of providing retroviral long terminal repeats (LTRs); (b) sequence required for reverse transcription; and (c) a D-type retroviral packaging sequence.

6.   A vector or construct as claimed in claim 5, wherein the sequence capable of providing LTRs is derived from a D-type retrovirus.

7.   A vector or construct as claimed in claim 5 or 6, wherein the sequence required for reverse transcription

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comprises a replication initiation site derived from a D-type retrovirus.

8.   A vector or construct as claimed in claim 5, 6 or 7, comprising retroviral donor (D) and acceptor (A) splice sites.

9.   A vector or construct as claimed in any one of claims 5 to 8 which is defective or deleted in *env*.

10.   A vector or construct as claimed in any one of claims 5 to 9 which is defective or deleted in *gag*.

11.   A vector or construct as claimed in any one of claims 5 to 10 which is defective or deleted in *pol*.

12.   A vector or construct as claimed in any one of claims 5 to 11, comprising exogenous nucleic acid.

13.   A vector or construct as claimed in claim 12, wherein the exogenous nucleic acid codes for a protein.

14.   A vector or construct as claimed in claim 13, wherein the protein is, or has qualitatively the same activity as, factor VIII, factor IX, adenoside deaminase, purine nucleoside phosphorylase, ornithine transcarbamylase, arginosuccinate synthetase, haemoglobin, α_1-antitrypsin, glucocerebrosidase, phenylalanine hydroxylase or hypoxanthine-guanine phosphoribosyl transferase.

15.   A vector or construct as claimed in claim 12, wherein the exogenous nucleic acid has, or is capable or having, a regulatory function.

16. A vector or construct as claimed in any one of
claims 12 to 16, wherein the exogenous DNA comprises a
suitable marker sequence.

5   17. A vector or construct as claimed in claim 16,
wherein the marker is a selectable marker.

18. A vector or construct as claimed in claim 17,
wherein the selectable marker is antibiotic resistance.

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19. A host cell containing a vector or construct as
claimed in any one of claims 5 to 18

20. An infectious particle comprising a vector as
15   claimed in any one of claims 5 to 18.

21. An infectious particle as claimed in claim 20 for
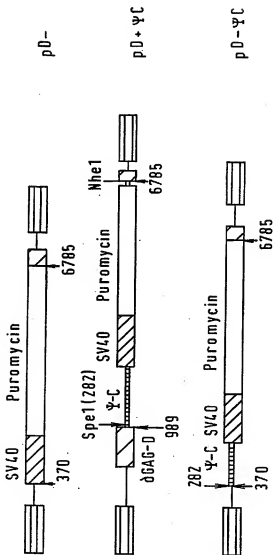use in medicine, particularly gene therapy.

FIG. 1
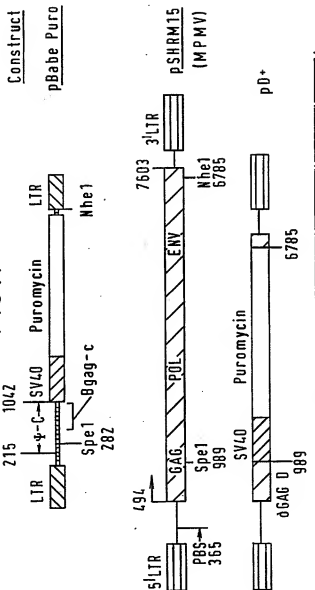
FIG. 1 (CONTD.)

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9317118	02-09-93	NONE	